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BEHAVIOURAL, HORMONAL AND HISTOPATHOLOGICAL CHANGES ACCOMPANYING THE OVERSIZED FOLLICLES IN CAMELS (*Camelus dromedarius*)

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ABSTRACT

This study was designed to investigate the effect of oversized follicles on the behaviour and hormonal concentrations in female dromedaries. The estrous pattern of 26 dromedaries with oversized follicles was recorded during the breeding season. Thirty–three ovarian pairs with preovulatory and oversized follicles were recovered and sectioned from slaughtered adult camels (n=33). Blood (10 ml) was collected from all females and follicular fluid from slaughtered females for estimation of reproductive hormones and nitric oxide (NO). Oversized follicles lead to infertility problems in dromedaries such as repeat breeding, nymphomania and anestrous. Serum progesterone (P_4) concentrations in repeat breeders with thin–wall oversized follicles (RB thin, n=10; 1411.50±93.39 pg/ml) and nymphomaniac with thin–wall oversized follicles (Nympho thin, n=8; 1710.00±107.74 pg/ml) were significantly ($P<0.05$) lower than that in anestrous animals with thick–wall oversized follicles (Anest thick, n=4; 2532.50±107.74 pg/ml). Serum estradiol (E_2) concentration was significantly ($P<0.05$) higher in Nympho thin (0.97±0.31 pg/ml) than Anest thick (0.30±0.08 pg/ml) camels. In Nympho–thin camels, serum testosterone (T; 39.75±4.85 pg/ml) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 173.93±9.75 pg/ml) concentrations were significantly ($P<0.05$) higher than both T concentration (17.20 ± 3.63 pg/ml) in RB thin and PG $F_{2\alpha}$ concentration (77.65±7.90 pg/ml) in RB thick camels (n=4). Serum NO concentrations in RB thin (2.49±0.03 μM) camels were significantly ($P<0.05$) higher than that in both RB thick and Anest thick camels. The oversized follicles lead to infertility problems in dromedaries, accompanied by changes in serum and follicular fluid reproductive hormones and NO concentrations.

Key words: Camel, follicular fluid, hormones, nitric oxide

In female dromedaries, the cystic ovaries were observed throughout the whole year, with variable percentages regarding both ovaries (Hussein *et al,* 2008). The follicular structures of dromedaries are classified into inactive ovaries (those containing follicles less than 3 mm in diameter), growing follicles (>3 to 9 mm in diameter), ovulatory follicles (10-19 mm in diameter) and oversized follicles (exceeding 25 mm in diameter) (range 40-64 mm), before they start regressing (Skidmore *et al,* 1996; Ali *et al,* 2010a; Skidmore, 2011). Follicles >30 mm in diameter are regarded as follicular cysts (Tibary and Anouassi, 1996). Follicle theca cysts have thin walls and fluctuate, while the contents are homogeneous and hypoechogenic (Ali *et al,* 2010a). Follicle lutein cysts or haemorrhagic cysts have thick, hard walls with non-homogenous and echogenic contents (Tibary and Anouassi, 2000). In llamas and alpacas, these oversized follicles may contain bloody fluid and are, therefore, termed haemorrhagic follicles that may become very large (up to 35 mm) and persist for a prolonged period (weeks) (Adams, 2007). The very large or haemorrhagic follicles do not appear to interfere with the growth of other smaller follicles on the same and contra-lateral ovaries and the ovarian activity may continue normally, thus, these large follicles do not constitute a major infertility problem in female camels (Tinson and McKinnon, 1992; Adams, 2007; Ali *et al,* 2010b; Skidmore, 2011). Although ovarian cysts have been described in dromedaries (El-Wishy, 1990; El-Khouly *et al,* 1990), the cystic ovary condition has not been well investigated as it has been in other domestic animals (Shawky *et al,* 2004; Ali *et al,* 2010a). In fact, the term "cystic ovaries" does not always apply to camels because a large proportion (30-40%) of females develop follicular cysts if not bred (Tibary *et al,* 2005). Ovarian cysts or oversized follicles have been found in the dromedary (El-Wishy, 1987; Tibary and Anouassi, 1996), bactrian camel (Bravo *et al,* 1993),

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llama and alpaca (Adams, 2007; Bravo *et al*, 1993). Incidence of ovarian cysts in female camels varied from 0.82 to 3.39 % (Musa, 1983; Omar *et al*, 1984; El-Wishy, 1989). The deficiency of luteinising hormone (LH) surge may be considered the main cause of cystic ovaries in camels (Jubb *et al*, 1993; Hegazy *et al*, 2004). Although some authors have suggested that ovarian cysts are a physiological variation of follicular dynamics (Tibary and Anouassi, 1997; Adams, 2007), others have claimed that camel ovarian cysts are pathological (Shawky *et al*, 2004; Ali *et al*, 2010a).

The objective of the current investigation was to study the behavioural, hormonal and histopathological alterations that accompanied the oversized follicles in the dromedary camels.

Materials and Methods

Animals and sampling

Twenty-six infertile female dromedaries (*Camelus dromedarius*) of 5-15 years of age, admitted to the Veterinary Teaching Hospital, King Faisal University, Kingdom of Saudi Arabia during the breeding season (November-April), were used in this study. Detailed previous breeding history and behaviour of the female camels were obtained. These females lived in groups with other female camels of different ages and near a mature male. A complete gynaecological examination of female dromedaries was performed via palpation and transrectal ultrasonographical (ALOKA SSD-500, Tokyo, Japan) examination (Anouassi and Tibary, 2013). These females had ovaries bearing oversized follicles of different diameters without any other ovarian structures like corpus luteum or normal–sized follicles. A 10 ml blood sample was collected via the jugular vein from each female into non–heparinised tubes and was centrifuged at 1500 g at 4°C for 10 min. Serum was harvested and stored at -20°C until analysis.

A total of 33 ovarian pairs were recovered from non-pregnant adult (7- 16 years of age) female camels (*Camelus dromedarius*) at a local abattoir in Al-Ahsa, Kingdom of Saudi Arabia. These paired ovaries were devoid of corpora lutea. Information about the reproductive status of these females was not available. A 10 ml blood sample was collected from each animal during slaughter. Immediately after collection, ovaries and blood samples were kept in an icebox and transported to the laboratory within one hour post-slaughter. Upon arrival at the laboratory, ovaries were washed twice in cooled 0.9% NaCl and left to dry. Two different follicle classes, based on follicle diameter (measured by Vernier caliper), were considered for puncture: oversized follicles of > 20 mm in diameter (n=21; Tibary and Anouassi, 1997) and preovulatory-sized follicles (dominant follicles according to the E2/P4 ratio) of 15-17 mm in diameter (n=12; Tinson and McKinnon, 1992). Follicular fluids were aspirated from all follicles using sterilised 22 gauge hypodermic needles and syringes. The follicular fluid and blood samples were centrifuged at 1500g at 4°C for 10 min. The supernatant was harvested and stored at -20°C pending analysis. Following aspiration, the oversized and preovulatory-sized follicles were sectioned and the obtained tissues were placed in 10% buffered formalin (Brandt and Manning, 1969) and processed for histopathological examination using paraffin wax. Four µm sections were cut and stained with haematoxylin and eosin (Schlafer, 2007).

Estimation of hormones and Nitric Oxide (NO) concentrations in serum and follicular fluid

Blood serum and follicular fluids progesterone (P_4) (pg/ml) were determined using EIA kits (Cayman Chemical Company, Ann Arbor, USA, Item No. 582601). The coefficients of variance (CV's) of the intra- and inter-assay were 7.3% and 16.4%, respectively. Oestradiol (E2) (pg/ml) was analysed by EIA kits (Cayman Chemical Company, Ann Arbor, USA, Item No. 582251). The CV's of the intra- and inter-assay were 7.4% and 10.7%, respectively. Testosterone (T) (pg/ml) was estimated using EIA kits (Cayman Chemical Company, Ann Arbor, USA, Item No. 582701). The CV's of the intra- and inter-assay were 4.4% and 7.7%, respectively. Human insulin like growth factor 1 (IGF-1; ng/ml) was assayed using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA, Catalog No. DG100, SG100, PDG100). The intra- and inter-assay CV's were 4.3% and 7.5%, respectively. $PGF_{2\alpha}$ (pg/ml) was analysed by EIA kits (Cayman Chemical Company, Ann Arbor, USA, Item No. 516011). The CV's of the intra- and inter-assay were 9.4% and 12.5%, respectively. NO (µM) was determined using Nitrate/Nitrite Fluorometric Assay kits (Cayman Chemical Company, Ann Arbor, USA, Item No. 780051). All assays were performed according to the manufacturer's directions, and the optical densities were measured using an ELISA Absorbance Microplate Reader (ELx 800TM, BioTek®, Highland Park, VT, USA) and Microplate Strip Washer (ELx800 TM, BioTek®, Highland Park, VT, USA).

Statistical analysis

Data are presented as means ± SEM. The female behaviour, hormones and NO were analysed by analysis of variance (ANOVA). The follicular fluid hormones and NO of oversized follicles and preovulatory follicles was compared by Student's *t-test* using SPSS statistical software program (2013), version 22.0.

Results

Table 1 showed that the classification of female camel behaviour coincided with the presence of oversized follicles. This classification revealed: (a) Repeat breeder female camel (female camel failed to conceive from 3 or more regularly spaced services in the absence of detectable abnormalities; Gustafsson and Emanuelson, 2002) with low–pitched male guttural humming sound and her ovary bearing thin wall (< 1 mm) oversized follicle (RB thin; n=10, 38.4%), (b) Repeat breeder female camel with low–pitched male guttural humming sound and her ovary bearing thick wall (> 1 mm) oversized follicle (RB thick; n=4, 15.4%; Fig 1), (c) Female camel experiencing nymphomania (abnormally excessive and uncontrollable sexual desire by a female) with low–pitched male guttural humming sound and her ovary bearing thin wall (< 1 mm) and oversized follicles (Nympho thin; n=8, 30.8%; Fig 2) and (d) Anestrous female camel showing signs of pregnancy (curls her tail dorsally) with low–pitched male guttural humming sound and her ovary bearing thick wall (> 1 mm) and oversized follicles (Anest thick; n=4, 15.4%; Fig 3). The male sound was expressed all the time by female camels. The diameter of oversized follicles ranged between 2.50 and 5.22 cm.

The mean concentrations of serum hormones and NO in female dromedaries that had oversized follicles are presented in table 1. Serum P_4 concentrations in RB thin and Nympho thin camels were significantly (P<0.05) lower than that in Anest thick camels. Serum E_2 concentration was significantly (P<0.05) higher in Nympho thin camels than in Anest thick camels. In Nympho thin camels, serum T and PG $F_{2\alpha}$ concentrations were significantly (P<0.05) higher than both T concentration in RB thin camels and $PGF_{2\alpha}$ concentration in RB thick camels. Serum NO concentrations in RB thin camels were significantly (P<0.05) higher than that in both RB thick and Anest thick camels (Table 1). The comparison of mean concentrations of hormones and NO in follicular fluid between slaughtered female dromedaries having either oversized or preovulatory follicles is presented in table 2. The mean concentrations of P_4, E_2, and NO in follicular fluid of the preovulatory follicles were significantly (P<0.05) higher than that in the oversized follicles. The diameter of oversized follicles in slaughtered female dromedaries ranged between 2.30 and 7.20 cm.

Histopathology of oversized follicles in slaughtered female dromedaries showed either thick or thin walled oversized follicles. Thick–walled oversized follicles appeared when the upper layer of the cystic wall was necrotic, granulosa cells were nearly absent, and the luminal contents of fibrinous strands and bands that attached to the wall were excessive (Fig 4). Thin–walled oversized follicles appeared with highly wrinkled or wavy cystic wall with excessively congested vasculature in the granulosa cell layer and underlying fibrous theca layers (Fig 5) or appeared with the granulosa cells nearly absent, with congestion and haemorrhages from the superficial small blood capillaries (Fig 6).

Table 1. Female camel behaviour in relation to types of oversized follicles and serum concentrations (mean ± SEM) of reproductive hormones and nitric oxide.

Serum parameters	Female behaviour and types of oversized follicles			
	Repeat breeder with low-pitched male guttural humming sound and thin wall oversized follicles (n=10) 38.4%	**Repeat breeder with load male guttural humming sound and thick wall oversized follicles (n=4) 15.4%**	**Nymphomania with low-pitched male guttural humming sound and thin wall oversized follicles (n=8) 30.8%**	**Anestrus (signs of pregnancy that curls her tail dorsally) with low-pitched male guttural humming sound and thick wall oversized follicles (n=4) 15.4%**
Progesterone (pg/ml)	$1411.50^{a} \pm 93.39$	$2207.50^{ab} \pm 113.29$	$1710.00^{a} \pm 107.74$	$2532.50^{b} \pm 107.74$
Oestradiol (pg/ml)	$0.47^{a} \pm 0.09$	$0.45^{a} \pm 0.12$	$0.97^{a} \pm 0.31$	$0.30^{b} \pm 0.08$
Testosterone (pg/ml)	$17.20^{a} \pm 3.63$	$44.00^{ab} \pm 6.12$	$39.75^{b} \pm 4.85$	$15.50^{ab} \pm 5.10$
IGF-1 (ng/ml)	189.00 ± 15.45	116.65 ± 46.27	179.25 ± 6.26	208.00 ± 1.22
Prostaglandin $F_{2\alpha}$ (pg/ml)	$211.34^{ab} \pm 34.43$	$77.65^{a} \pm 7.90$	$173.93^{b} \pm 9.75$	$105.75^{ab} \pm 2.96$
Nitric oxide (μM)	$2.49^{a} \pm 0.03$	$1.91^{b} \pm 0.02$	$2.37^{ab} \pm 0.10$	$2.06^{b} \pm 0.07$

Means with different superscripts in the same row are significantly different at P<0.05.

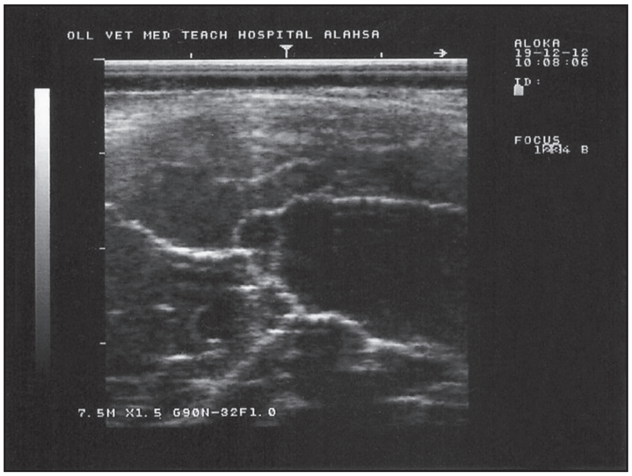

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Fig 1. Ultrasonography of oversized follicle (5.40 cm diameter) with thick wall and fibrinous strands.

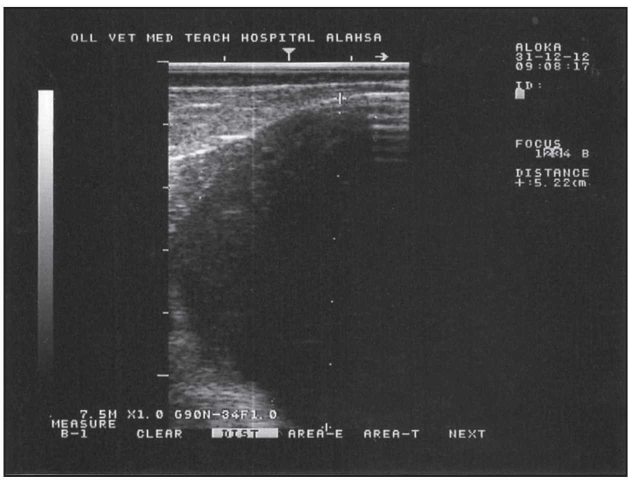

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Fig 2. Ultrasonography of oversized follicle (5.22 cm diameter) with thin wall and hypo-echoic contents.

Discussion

In the present study, the percentage of repeat breeder female camels with oversized follicles was 53.80%. On the same basis, repeat breeder syndrome has been recorded in 66.67% of female dromedaries bearing ovarian cysts (Ali *et al,* 2010a). Thin–walled oversized follicles were 69.20%. In a previous study, follicular cysts were recorded as 53.06% (Shawky *et al,* 2004). Female camels bearing thick–walled oversized follicles on their ovaries were 30.80% of studied oversized follicles. However, luteal cysts constitute 10.20% of ovarian cysts in camels (Shawky *et al,* 2004). Female dromedaries with oversized follicles and signs of pregnancy (15.40%) showed a dorsal curling up of their tails. However, this response could be also observed in animals with progesterone secreting cysts (Monaco *et al,* 2015).

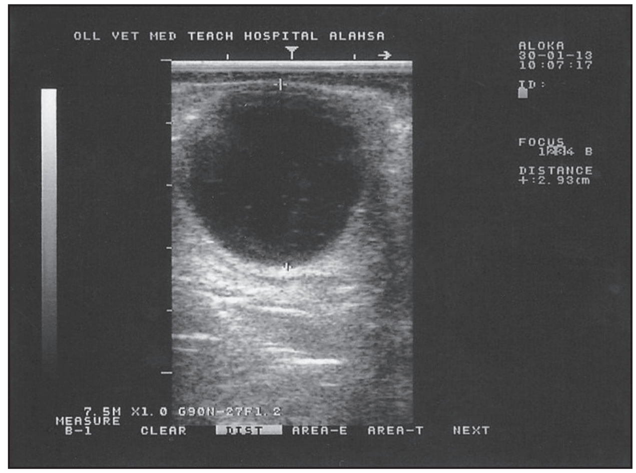

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Fig 3. Ultrasonography of oversized follicle (2.93 cm diameter) with thick wall and hypo-echoic contents.

In the present study, the repeat breeder female camels having thick–walled oversized follicles with fibrous strands on their ovaries constituted 15.40% of studied large follicles. Follicles that grow beyond 2.0 cm (4.0 – 6.4 cm) in diameter do not ovulate, but these follicles develop echogenic strands of fibrin as they degenerate (Skidmore *et al,* 1996; Skidmore, 2011). In contrast to our previous study it seems probable that these oversized follicles were pathologic and appeared to interfere with the growth of other follicles, constituting a major infertility problem in female camels in the forms of repeat breeding, nymphomania and anestrous (Ghoneim *et al,* 2013).

In the current study, serum P_4 concentrations in RB thin and Nympho thin camels were lower than that in Anest thick camels. However, serum P_4 concentrations are higher in the dromedary follicular cyst than in the luteal cyst (3.27 Vs 1.66 ng/ml, respectively; Hegazy *et al,* 2004). Lower values of

Table 2. Concentrations (mean ± SEM) of reproductive hormones and nitric oxide in follicular fluid from female camels bearing either oversized follicles or preovulatory follicles.

Parameters	Abattoir oversized follicular fluid (n=21)	Abattoir preovulatory follicular fluid (n=12)
Progesterone (pg/ml)	$2409.72^a \pm 64.37$	$2638.00^b \pm 27.10$
Oestradiol (pg/ml)	$522.69^a \pm 100.22$	$874.09^b \pm 28.53$
IGF-1 (ng/ml)	124.67 ± 8.98	150.36 ± 12.56
Prostaglandin $F_{2\alpha}$ (pg/ml)	386.97 ± 82.69	382.09 ± 91.05
Nitric oxide (µM)	$1.85^a \pm 0.12$	$3.91^b \pm 0.14$

Means with different superscripts in the same row are significantly different at P<0.05.

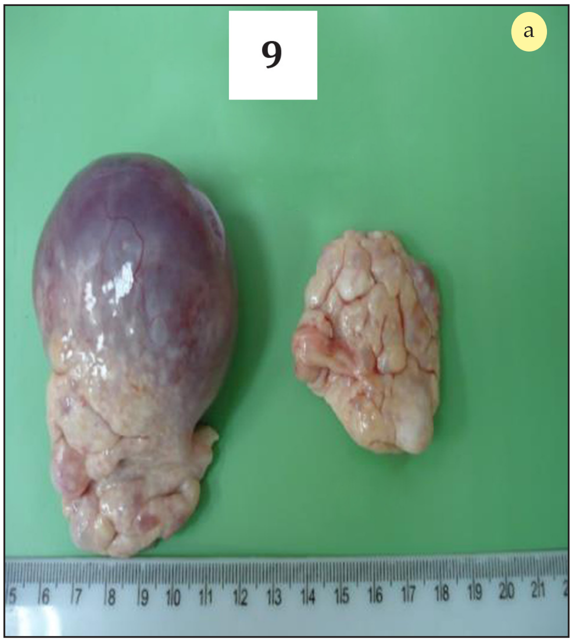

Fig 4a. Left ovary bearing oversized follicle appeared thick wall, bloody and multi-cavities with 5.7 cm in diameter. Right ovary has no structure.

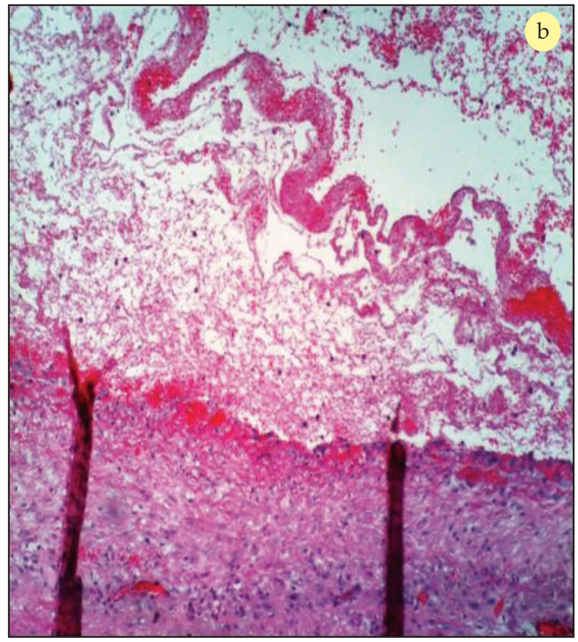

Fig 4b. Histopathology of left ovarian bloody oversized follicle (5.7 cm): The upper layer of the cystic wall appeared necrotic and nearly absence of granulosa cells and excess of luminal contents of fibrinous strands and bands that attached to the wall. H and E X=250.

Fig 5a. Left ovary bearing oversized follicle appeared thin wall with 4 cm in diameter and light red. Right ovary has no structure.

Fig 5b. Histopathology of thin walled and light red oversized follicle (4 cm) in left ovary: Highly wrinkled or wavy cystic wall appeared with excess of congested vasculature in the granulosa cell layer and underlying fibrous theca layers. H and E X=63.

serum P_4 concentrations have been recorded (0.0089 Vs 0.0093 ng/ml, respectively) for follicular and luteal cysts (Hussein *et al*, 2008) and for oversized follicles (0.53 ng/ml) (Ghoneim *et al*, 2013) in female camels. However, in sows, there is no effect of growing or decreasing number of ovarian cysts on concentrations of plasma P_4 (Szulanczyk-Mencel *et al*, 2010). In the follicular fluid, P_4 concentrations from oversized follicles were lower than that from the preovulatory follicles. However, this difference has not been found between the concentrations of P_4 in follicular fluid from cyst-like follicles and preovulatory follicles (Ghoneim *et al*, 2013). Nevertheless, in buffaloes, greater concentrations of P_4 have been reported in the cysts than normal preovulatory follicles (Goralczyk *et al*, 1992). In sows, the cystic fluid of animals with

Fig 6a. Left ovary has no structure. Right ovary is an oversized follicle 5.3 cm in diameter, thin wall and reddish color.

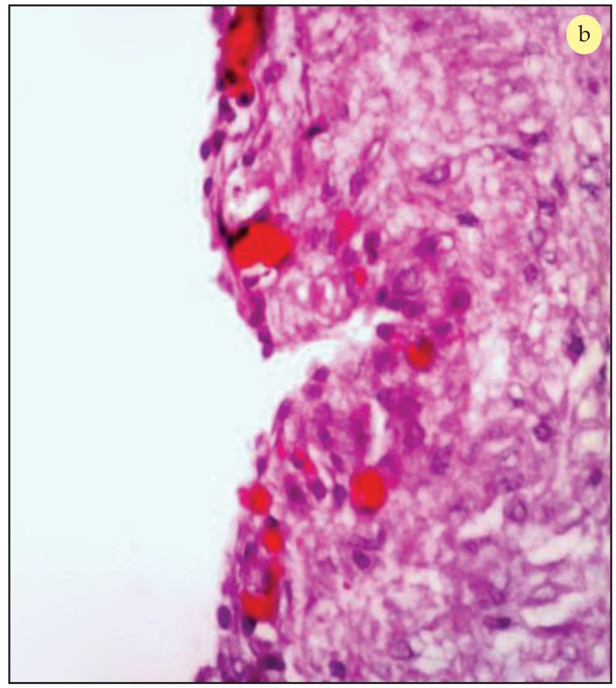

Fig 6b. Histopathology of thin walled and reddish oversized follicle (5.3 cm) in the right ovary: Nearly absence of the granulosa cells with congestion and haemorrhages from the superficial small blood capillaries. H and E X= 400.

oligocystic ovaries had a significantly (P< 0.001) higher P_4 concentration in comparison to polycystic animals (Ebbert *et al*, 2007).

In this study, serum E_2 concentration was higher in Nympho thin than Anest thick camels. However, there are no significant differences of serum E_2 between dromedary animals which have cyst-like follicles and those bearing preovulatory follicles on their ovaries (Ghoneim *et al,* 2013). Moreover, there is no line of demarcation in serum E_2 concentrations between female camels having either follicular or luteal cysts (Hegazy *et al,* 2004; Hussein *et al,* 2008). Previous studies reported no difference between blood E_2 concentrations of cystic and normal estrus in both cows (McNatty *et al,* 1984) and sows (Szulanczyk-Mencel *et al,* 2010). Follicular fluid E_2 concentrations from the preovulatory follicles were higher than those from oversized follicles. Parallel findings have been recorded in dromedary camels (Ghoneim *et al,* 2013) and cattle (Glencross and Munro, 1974; Gustafsson and Emanuelson, 2002). However, there is no significant variation in the concentration of E_2 between cystic and preovulatory follicles in buffalo (Goralczyk *et al,* 1992). Ovaries of cows with COD exhibited altered estrogen receptors expression compared with that in normal animals (Salvetti *et al,* 2007). In buffaloes with ovarian cysts, serum estrogen is significantly (P<0.5) increased, while progesterone is significantly (P<0.5) decreased (El-Sakkar *et al,* 2008).

In the present work, serum T and $PGF_{2\alpha}$ concentrations were significantly (P<0.05) higher in the Nymph thin camels than in both T concentration in RB thin and $PGF_{2\alpha}$ concentration in RB thick camels. Large amounts of P_4 are produced from granulosa and theca cells of bovine follicles which serve as a precursor for androgen and subsequently estrogen production (Homeida *et al,* 1988). The production of proteolytic enzymes is enhanced by P4 that promote the rupture of follicles at ovulation (Skidmore *et al,* 1994). In sows, T and E_2 levels in plasma and in cystic fluid of polycystic animals were significantly (P<0.01) higher in comparison to oligocystic animals, while P_4 concentration was almost the same (Szulanczyk-Mencel *et al,* 2010). In oligocystic ovaries, T in cysts exceeded the E_2 levels, whereas in polycystic ovaries the situation is reversed (P < 0.001; Ebbert *et al,* 2007).

In the present study, serum NO concentration in RB thin camels was higher than that in both RB thick and Anest thick camels. A decrease in serum NO concentrations are found in infertile cows affected with ovarian cysts (Mutlag *et al,* 2015). Serum NO values are low (P<0.01) in buffalo-cows suffering from parasitic infestation as compared to healthy animals (El-Khadrawy *et al,* 2008). The mean concentrations of NO in follicular fluid of the preovulatory-sized follicles were higher than that in the oversized follicles. In buffaloes, follicular cysts were characterised by greater ($P<0.01$) concentrations of NO and P_4 than that of preovulatory-sized follicles (Khan *et al,* 2011). These greater P_4 concentrations inhibit the onset of LH surge resulting in the formation of follicular cysts. In addition, it declares the role of intra-ovarian regulators, such as NO, in development of the condition (Khan *et al,* 2011). Nitric oxide was found to be involved in the formation of hCG-induced murine follicular cysts (Nemade *et al,* 2002) and in the pathophysiology of polycystic ovary syndrome in rats (Hassani *et al,* 2012). Ovulation is a physiological process that depends on the coordinated activity of gonadotropins and steroid hormones, as well as inflammatory mediators such as NO (Khodaei *et al,* 2009). Histopathology of studied oversized follicles showed necrosis of the follicle wall, the near absence of granulosa cells, and excess luminal contents of fibrinous strands that attached to the wall. Sometimes, congestion and haemorrhages occur from the superficial blood capillaries. Histological studies of large follicles (>3 cm diameter) revealed that the granulosa cells had degenerated and become reduced to a single layer and the thecal layer thinned and became less distinct from the adjacent stroma (Skidmore, 2011). In buffaloes, the examined ovaries showed either thick walled follicular cysts or leutein cysts with luteinised granulosa cells, hyperplastic theca-interna and theca-externa besides atretic follicles and hyperplastic lining of other follicles (El-Sakkar *et al,* 2008).

In female dromedaries, the oversized follicles were accompanied with infertility problems in the form of repeat breeding, nymphomania and anestrus. Although, the peripheral blood reproductive hormones and NO concentrations were affected by the presence of the oversized follicles, the follicular fluid concentrations of P_4, E_2 and NO were significantly higher in the preovulatory-sized follicles than in oversized follicles.

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